Grading Pennsylvania Cigar-leaf Tobacco

First step in the case fermentation process.

Reid, J. J. et al

BULLETIN 363

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Studies on the FERMENTATION OF TOBACCO

2. Microorganisms Isolated from Cigar-leaf Tobacco

THE PENNSYLVANIA STATE COLLEGE
SCHOOL OF AGRICULTURE AND EXPERIMENT STATION
STATE COLLEGE, PENNSYLVANIA

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PENNSYLVANIA AGRICULTURAL EXPERIMENT STATION

BULLETIN 363* STATE COLLEGE, PA. JUNE 1938

Studies on the Fermentation of Tobacco

2. Microörganisms Isolated from Cigar-leaf Tobacco

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IN previous publications *(10, 11, 12, 13)* the authors have shown that the reproduction of certain microörganisms is associated with the successful fermentation of cigar-leaf tobacco and that the leaf exhibits a characteristic microflora, the predominating forms of which are determined by the substrate-moisture-oxygen-temperature relationships maintained during the course of fermentation.

The studies reported in this bulletin represent an attempt to describe morphologically and physiologically the microörganisms found in significant numbers on cured and fermenting cigar-leaf tobacco, which play an important part in the quality of the fermented product.

MATERIALS AND TECHNIQUE

The observations reported in this bulletin are based on the study of 2844 pure culture isolations of microörganisms from 354 samples of cured and fermenting cigar-leaf tobacco, table 1. The tobaccos from which these isolations were made were representative of the cigar-filler and binder types produced in Pennsylvania, Wisconsin and Ohio.

Isolations of cocci and aerobic spore-forming bacteria were made from dilution plates prepared in the routine examination of tobacco. In many cases isolations were made from a single colony of each colony type found on the plates at the time the count was made; in other cases isolations were made from all colonies appearing on the plates in the dilution counted. Although the first method furnishes a culture collection suitable for the identification

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of the various forms appearing during the course of the fermentation, the second method is essential to a study of the physiological adaptations taking place as the fermentation proceeds, and observations of such a nature are based on this second method of collecting cultures.

Table 1.—Pure culture isolations from different samples of cigar-leaf tobacco.

Isolated From	Number of Samples	Number of Isolations of: Cocci	Aerobic Spore-formers	Other Bacteria	Fungi	Total
Cured Pennsylvania, 1937 crop	15	7	109	17	210	343
Cured Pennsylvania, 1936 crop	112	5	419	3	317	744
Cured Wisconsin, 1937 crop	9	3	57	2	141	203
Case fermenting Pennsylvania, 1936 crop	22	124	71		35	230
Bulk fermenting Pennsylvania, 1930 crop	41	56	145	1		202
Laboratory fermenting Pennsylvania, 1936 crop	76	59	233	2	7	301
Bulk fermenting Wisconsin, 1931 crop	36	61	139	5	43	248
Laboratory fermenting, Wisconsin 1937 crop	31	385	42	22	19	468
Bulk fermenting Ohio, 1932 crop	10	11	40		3	54
Case fermenting Maryland, 1936 crop	2		4		47	51
Totals	354	711	1259	52	822	2844

Crystal violet in an appropriate concentration, usually 1:200,000 was added to the nutrient agar used in plates made for the purpose of determining the presence of gram-negative bacteria. All bacterial colonies appearing on these plates were examined microscopically and isolations were made from all colonies found to be gram-negative as well as from colonies the Gram reaction of which at this time was not well defined. In further efforts to determine the presence of gram-negative bacteria, crystal violet was added to beef-extract peptone glucose agar. Dilution plates were poured with this medium and colonies picked as previously described.

Isolations of anaerobes were made from deep tubes of crystal violet beef-extract peptone glucose agar. With a concentration of gentian violet of 1:200,000 the colonies of *Micrococcus* and *Bacillus* ordinarily far outnumbered the colonies of the genus *Clostridium* in this substrate, rendering isolation of the anaerobes somewhat difficult. The pyrogallol method of obtaining anaerobic conditions in the tubes was used in some instances.

Cellulose-fermenting species of *Clostridium* were isolated by the enrichment method, using the medium of Omeliansky and incubating duplicate tubes at 37 and 55° C.

Morphological identifications of fungi were made from the growth on glucose–peptone–acid agar. Isolations were made from colonies not easily identified in this manner and were the subject of further study.

Cultural studies useful in the classification of the several bacterial forms found were made in all cases. These studies included all morphological and physiological information found to be of value in comparing these isolations with species and other groupings described by Bergey *(2)*, Lehman and Neuman *(9)*, Hucker *(5)*, and Smith and Clark *(16)*.

Micrococcus isolations that showed a tendency to appear in grapelike clusters were investigated serologically according to the method of Julianelle *(7)*.

MICROCOCCI OF CIGAR-LEAF TOBACCO

The presence of gram-positive cocci upon most samples of cured and fermenting cigar-leaf tobacco is easily demonstrated. Although greatly outnumbered by the spore-formers on the cured leaf, the cocci have been found to multiply rapidly during the early stages of active fermentation, frequently reaching a number by plate count, in excess of 1,000,000,000 cells per gram. As a rule their numbers decline rapidly following this maximum and it becomes increasingly difficult to prove the presence of this coccus form in the later stages of the process.

Morphological classification.—The cocci found upon cigar-leaf tobacco may be roughly classified upon the basis of morphology, in three types:

TYPE I.—Cells small, occurring in grapelike clusters.

TYPE II.—Cells somewhat larger than in Type I and occurring singly, in pairs, and in small clusters.

TYPE III.—Cells much larger than in either of the preceding types, somewhat oval, occurring singly, in pairs and in clusters. Such a classification, however, is abritrary and extremely unsatisfactory, inasmuch as more than half the isolations occupy intermediate positions with respect to the types described. In fact, the culture collection of 711 cocci isolated from tobacco ranges from the extreme of Type I to the extreme of Type III with no clear-cut lines of demarcation separating the types. Type I has ordinarily been found to be associated with the earlier stages of the fermentation, Type II with that period in which the rise in numbers is most rapid and Type III with the period of highest bacterial count.

Classification upon the basis of pigment production reveals that almost all isolations from tobacco undergoing a satisfactory fermentation are non-pigmented forms. A few pigment formers have

been isolated from cured tobacco and from tobacco held at a moisture content too low for active fermentation.

Gelatin liquefaction and activity in litmus milk offer a basis for the classification of these organisms which might appear satisfactory. A cultural description of the five types and one heterogeneous group differentiated in this manner with the number of isolations falling into the various types is given in table 2.

Table 2.—Classification of 711 cultures of micrococci isolated from cured and fermenting tobacco, upon gelatin liquefaction and action in litmus milk. 14 day incubation.

Action Upon	Type I	Type II	Type III	Type IV	Type V	Group VI
Gelatin	Crateriform	Crateriform	Crateriform	No liquefaction	No liquefaction	Rapid, other than Crateriform
Litmus milk	Acid curd	Acid, no curd	No change	Acid, no curd	No change	Proteolysis
Dextrose	+	+	+	+	—	±
Sucrose	+	+	+	+	—	±
Lactose	+	+	±	+	—	±
Mannitol	+	+	+	—	—	—
Growth at 55° C	—	—	—	—	+	—
Number of isolations	189	151	73	51	204	43

In this biochemical separation, as in morphological separation, classification rests upon purely arbitrary decisions. An incubation period of two weeks was the basis of the separation upon gelatin and litmus milk reactions. Any other incubation period would give different groupings of the cultures; shorter incubation would move many of Type I into Type II, many of Type II into Type III, etc. On the other hand a longer incubation woud move many of the Type IV and V cultures into Types I, II or III, some Type III cultures into Type II and some Type II cultures into Type I. In other words, cultures classified in one type often differ from those in another type not in the inherent ability to carry out a certain reaction but in the speed with which the particular cultures are able to bring it about.

Inasmuch as attempts to classify cultures of micrococci obtained from tobacco suggested the possibility of physiological adaptations to an ever-changing environment, a series of 217 isolations of micrococci from a single small experimental bulk of fermenting tobacco were made in the course of 18 days. Dilution plates were poured with sufficient interpolations in the series to obtain each day a plate

with from 20 to 40 colonies. Isolations were made from all colonies appearing on these plates and a study was made of the morphological and physiological characteristics of the cultures of cocci.

The morphological classification of the daily isolations of cocci are shown in figure 1. Although the isolations made the first day of the fermentation were easily classified as staphylococcus-like in appearance, and most of those isolated toward the end of the 18-day

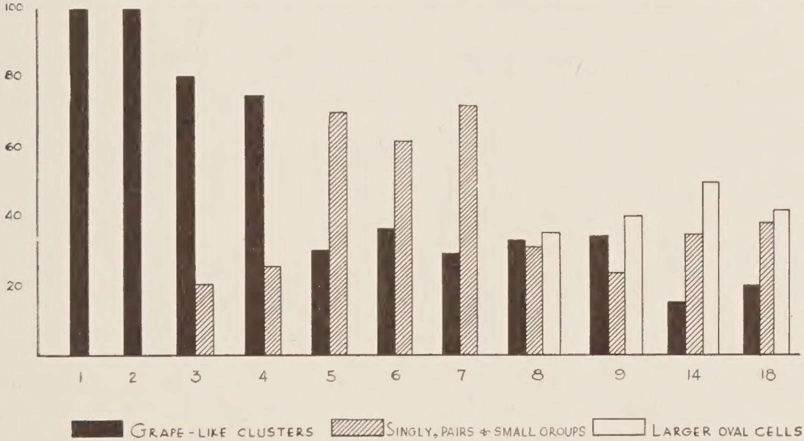

Fig. 1.—Morphological classification of micrococci isolated from a bulk of fermenting tobacco.

fermentation were obviously large oval-celled cultures, many if not most of the cultures isolated from the third to ninth days were classified arbitrarily as these isolations represented morphological intermediates. The two extremes are shown in figures 2 and 3.

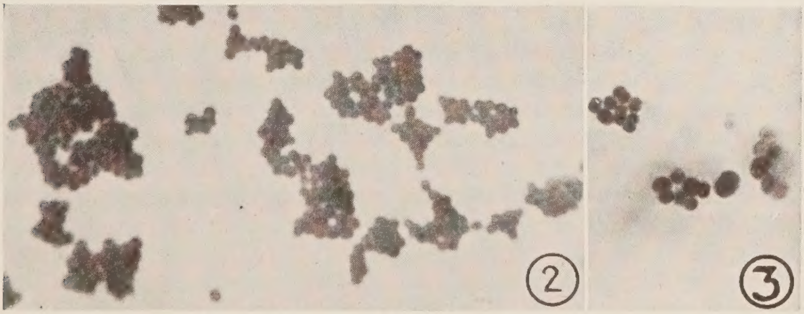

Fig. 2.—Micrococci isolated in the initial stage of cigar-leaf fermentation. The grape-like clusters are characteristic of the cocci at this stage. (950 X)

Fig. 3.—Micrococci isolated at the most active stage of fermentation. The large oval cells are characteristic of the cocci at this stage. (950 X)

Figure 1 emphasizes the relation of morphological type to the stage of the fermentation, confirming previous conclusions made on the basis of more or less random isolations. A study of the 217 cultures reveals that the change is indeed a gradual one.

Classification by gelatin liquefaction and action in litmus milk.—The description of the five types and one heterogenous group listed in table 3 is presented in table 2. Table 3 reveals a daily change in the biochemical activities of the predominant cocci of the bulk.

Table 3.—Cultural classification of 217 cultures of micrococci isolated from a bulk of fermenting tobacco during a period of 18 days.

Day of Isolation	Type I	Type II	Type III	Type IV	Type V	Group VI
First	27	3	0	0	0	0
Second	14	6	0	0	0	0
Third	19	4	1	0	0	0
Fourth	3	4	0	0	0	0
Fifth	5	3	3	0	0	0
Sixth	6	3	26	3	0	0
Seventh	3	2	2	0	0	0
Eighth	0	8	7	4	1	0
Ninth	0	6	0	2	10	3
Fourteenth	0	1	0	2	10	5
Seventeenth	0	4	0	1	15	0
Total cultures	77	45	39	12	36	8

Starting with an active type of organism capable of liquefying gelatin, producing an acid curdling of milk and fermenting several of the common sugars, the change proceeds by degrees to a form incapable of liquefying gelatin, producing no change in milk and unable to ferment the common carbohydrates. Gelatin liquefaction of these organisms is shown in figure 4.

Figure 1 and table 3 show that there is some relation between morphology and cultural characteristics. Micrococci occurring in grape-like clusters usually fall in Types I or II of table 2, and the larger oval-celled forms more often fall in the relatively inert Type V. But the relationship is not an exact one and morphological types are scattered throughout the six biochemical groupings.

The speed of the reaction rather than the reaction itself determines, in many cases, the classification made in tables 2 and 3. The speed of these reactions is of interest in this connection, particularly with reference to gelatin liquefaction and changes in litmus milk.

That the changes in the ability to liquefy gelatin as the fermentation of the bulk proceeded are rather gradual in nature is shown in table 4. All gelatin liquefiers among the 217 isolations made from this experimental bulk were included in table 4 with the exception of the eight cultures classified in Group VI, table 3. These cultures

liquefied the gelatin rapidly, in either saccate or stratiform manner and apparently bear little relation to the other 209 isolations from the bulk.

All micrococcus isolations from the bulk have been cultured in litmus milk and gelatin several times and the results have agreed extremely well. The cultures have not been held within the laboratory for sufficient time to make any statement as to retention of initial characteristics on prolonged laboratory cultivation.

Table 4.—The speed of gelatin liquefaction by micrococci isolated during the course of 18 days from a fermenting bulk of cigar-leaf tobacco.

Day of Isolation	Per Cent of Cocci Giving Crateriform Gelatin Liquefaction	Days of Incubation at 20°C Necessary to Obtain Evidence of Liquefaction
First	100	1– 2 days
Second	100	2 days
Third	100	3 days
Fourth	100	3– 4 days
Fifth	100	4 days
Sixth	90	4– 5 days
Seventh	80	5 days
Eight	80	5– 6 days
Ninth	50	10 days
Fourteenth	38	10 days
Seventeenth	20	10–12 days

The litmus milk reactions of this group of cultures present a picture strikingly similar to that presented by the gelatin characteristics. As daily isolations were made there was first noted a gradual change in the length of time required to coagulate milk, followed by an increasingly larger percentage of isolations incapable of coagulation. This, in turn, was accompanied and followed by changes in the ability to produce an acid reaction in milk, the length of time required for such acid production becoming increasingly longer. Eventually most isolations were unable to produce changes in milk during the 14-day period of incubation. With regard to the utilization of carbohydrates and alcohols as sources of energy, the loss of the ability to utilize mannitol was first noted, followed in turn by the degree of acidity produced in lactose, the length of time required to produce acidity in any of the carbohydrate media employed and finally the loss of the ability to ferment the common carbohydrates.

Two theories may be advanced to account for the changing morphological and physiological characteristics of the isolations of micrococci from tobacco undergoing fermentation. It may be assumed that the fermentation is characterized by a succession of

species or physiological types of cocci, one species replacing another in rapid succession; or that the isolations merely reflect physiological adaptations of a species to a constantly changing environment. Perhaps some of the changes noted may actually represent succession of species and others physiological adaptation.

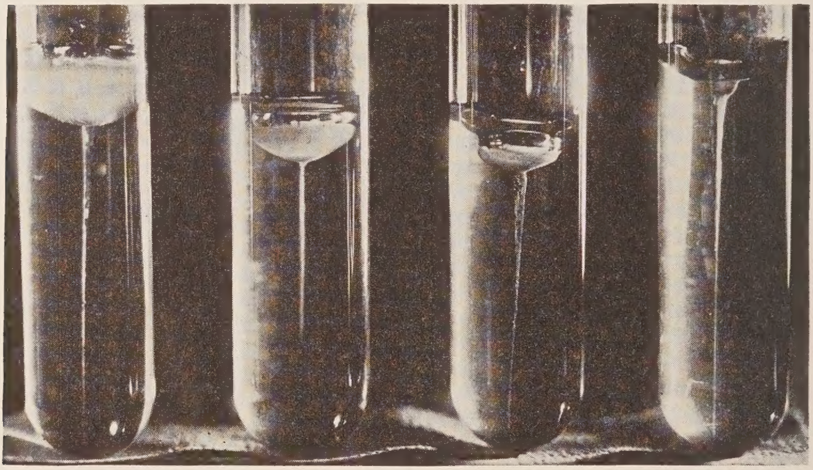

Fig. 4.—Gelatin tubes inoculated with micrococci isolated at successive stages in the fermentation. Incubation 14 days. Tube at extreme left inoculated with isolation obtained on the first day of fermentation; tube on the extreme right inoculated with isolation made the fourteenth day of the fermentation. Note loss in speed with which liquefaction of gelatin takes place.

In further efforts at classification other cultural characteristics of the 209 isolations listed as Types I to V inclusive in table 3 were determined. All proved able to reduce nitrates to nitrites, none was able to grow on the mono-ammonium phosphate medium of Hucker *(5)*, none was able to utilize urea and growth on potato was slow, restricted and yellow to white in color. The precipitin test of Julianelle *(7)* showed no relation between these isolations and either the A or B groups of staphylococci.

Although it is not suggested that this collection of cultures represents species hitherto undescribed, nevertheless the schemes of classification of Hucker *(5)* and of Bergey *(2)* offer little help in the classification of the various types of this collection. The scheme of Lehmann and Neumann *(9)* is more useful. Types I to IV, table 3, might be considered as varieties of *Micrococcus candicans* as described by Lehmann and Neumann *(9)*. Type V might be considered as closely related to *Micrococcus viticulosus* of the same

authors. There is much more latitude in the definition of a species by Lehmann and Neumann *(9)* than by either Hucker *(5)* or Bergey *(2)*. However, in the consideration of such a tentative classification, the morphological characteristics of *Micrococcus viticulosus*, although corresponding with the characteristics of a majority of the forms classified in table 3, Type V, are found to a lesser extent in most of the other types of table 3.

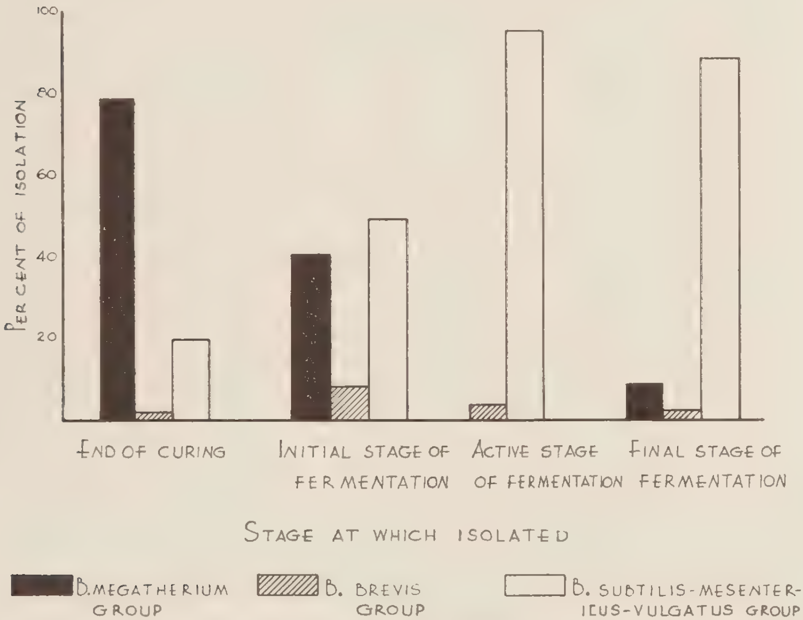

Fig 5.—Relative numbers of the three groups of aerobic spore-formers found on cigar-leaf tobacco at various stages in the processing.

The presence of micrococci on fermenting tobacco has been noted by Schloesing *(14)* and Suchsland *(17)*. Koning *(8)* described an organism that he termed "*Diplococcus tabaci*" found on fermenting tobacco. This was described, however, as gram-negative. Schmidt *(15)* observed cocci on tobacco of poor quality. Johnson *(6)* found staphylococci on fermenting leaves. Giovannozzi *(4)* found cocci on the fermenting product and reports that Rossi and Volganow also found this form associated with the fermentation. Giovannozzi's description of the cocci found by Rossi is of particular interest. Termed *Micrococcus nicotianae*, the organism was oval, .9 x 1.2 micra, gram-positive, facultative, producing no change in milk and failing to ferment sucrose and to liquefy gelatin. The

organism described by Rossi as reported by Giovannozzi *(4)* bears a striking similarity to Type V, table 3.

Occurring frequently in numbers exceeding 1,000,000,000 per gram, the importance of micrococci in the fermentation of cigar-leaf tobacco cannot be denied. Inasmuch as these have been found

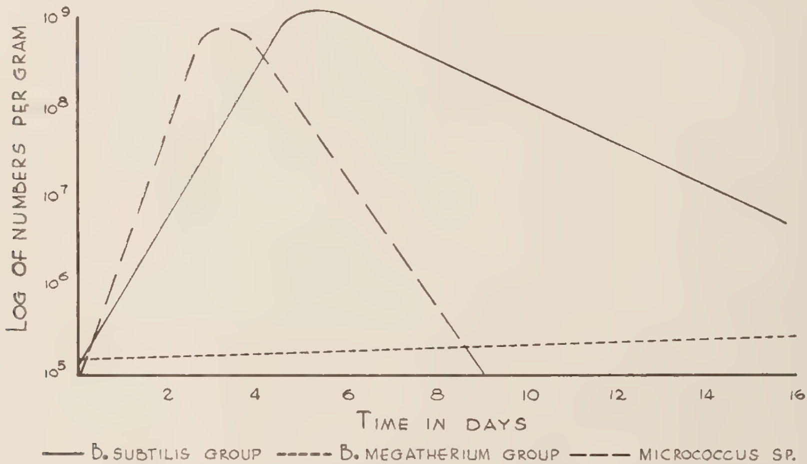

Fig. 6.—Prepared from data obtained in a laboratory fermentation, showing the numbers of the *B. subtilis* group, *B. megatherium* group and cocci found throughout the course of 16 days. In a general way this corresponds with data obtained from commercial fermentation of the rapid type.

upon tobacco of the highest quality as well as upon inferior grades and in fermentations pronounced entirely satisfactory by the manufacturer, micrococci must be considered important members of the normal flora of the fermenting cigar-leaf.

BACILLI OF CIGAR-LEAF TOBACCO

All samples of cigar-leaf tobacco examined in this laboratory have shown the presence of aerobic spore-formers. The numbers found have varied from a few thousand per gram, in some samples of cured tobacco and improperly fermenting tobacco, to counts in excess of 1,000,000,000 per gram in samples taken from the active stages of fermentation.

More than 1200 pure culture isolations of this group of bacteria were studied. The scheme of Smith and Clark *(16)* has been found useful in an attempt to classify these isolations. Classified according to this scheme the isolations have fallen into three groups, the *Bacillus megatherium–cereus* group, the *B. subtilis–mesentericus–vulgatus* group and the *B. brevis* group, table 5, and figure 5.

Organisms of the *B. megatherium–cereus* group liquefy gelatin rapidly, produce no gas in carbohydrate media, store reserve materials which may easily be demonstrated in the cell, lose their gram-positive characteristics within a short time following transfer, produce a rather large cell containing central to eccentric spores, frequently are found in rather long chains, grow best in the temperature range of 25 to 35° C and fail to grow at a temperature of 50° C.

Table 5.—Classification of the aerobic spore-forming bacteria isolated from cigar-leaf tobacco.

ORGANISM		STAGE AT WHICH ISOLATED				TOTAL
		End of Curing	Fermentation			
			initial stage	*active stage*	*final stage*	
B. megatherium group		463	57	0	21	541
B. brevis group		9	12	11	5	37
B. subtilis–mesentericus–vulgatus group	Type A	21	10	65	42	138
	Type B	15	16	30	22	83
	Type C	37	21	155	16	229
	Type D	40	23	35	133	231
Total		585	139	296	239	1259

A microphotograph of a member of this group is presented in figure 7. This is the characteristic bacterial group of cured tobacco, usually outnumbering all other bacterial groups on plates inoculated from the cured leaf. It has not been found to reproduce to any great extent during the normal fermentation, although it may often be found in slightly greater numbers at the end of the fermentation than at the beginning. More than one species of this group is found, as a rule, in the plating of cured tobacco. *B. megatherium* and *B. cereus* are most often encountered although *B. mycoides* and other members of the group are occasionally noted in the plating of cured tobacco. As shown in table 5, members of this group have not been found on plates made during the active stages of fermentation, too many members of *Micrococcus* and the *B. subtilis–mesentericus–vulgatus* group being present at that stage for the appearance of the *B. megatherium–cereus* group on countable plates. On the basis of this study it is concluded that any rôle the *B. megatherium* group may play in the fermentation is a minor one.

Organisms of the *B. subtilis–mesentericus–vulgatus* group liquefy gelatin, do not produce gas from carbohydrates, do not store reserve fat, produce acetyl-methyl-carbinol, usually retain gram-positive character much longer than do members of *B. megatherium–cereus* group, produce a rather small cell as contrasted with many members of the genus, spores are ordinarily central to

eccentric, cells often found singly, in pairs or short chains, and grow over a rather wide range of temperature, many forms growing at 55° C. This is the characteristic spore-forming group of fermenting tobacco. During the course of the actual fermentation of the tobacco this group has never been found to be outnumbered by any form other than micrococci and then for only a limited period.

Table 6.—Distinguishing cultural characteristics of the four types of the *B. subtilis-mesentericus-vulgatus* **group found on fermenting cigar-leaf tobacco.**

	Type			
	A	B	C	D
Growth on potato	Very slow, pink, glistening, vesicular	Rapid growth, pink, smooth	Dark gray to brown, smooth or rough	Wrinkled, without pigment or pink
Growth on ferric ammonium citrate tryptone agar	Pink, vesicular, spreading	Rough, dry, netted, cream colored		
Liquefaction of gelatin	Crateriform becoming stratiform	Saccate	Saccate	Saccate
Litmus milk	Coagulated without acid, serum zone, slow proteolytic action			Rapid complete digestion
Galactose	Ferments with pellicle	Variable	Variable	Does not ferment
Raffinose	Does not ferment	Ferments	Does not ferment	
Growth at 55° C	Good	Variable		None

All isolations of this group from tobacco grow well on a synthetic substrate utilizing an ammonium salt as the source of nitrogen. Energy is readily supplied by the citrate and malate ions and by a number of the common carbohydrates. Cultivation upon laboratory media shows that the isolations belonging to this group may be roughly classified into four types. The incidence of these four types, termed A, B, C and D for convenience, is shown in table 5 and the distinguishing characteristics in table 6.

Type A of the *B. subtilis–mesentericus–vulgatus* group is characterized by ability to grow well at 55° C, formation of a pellicle on many substrates in which the other three types of this group fail to form pellicles, peculiar pink vesicular growth on ferric ammonium citrate tryptone agar, and crateriform to stratiform liquefaction of gelatin. The colony formation of this organism

when cultivated at 45° C or lower is peculiar, the colony appearing as a rosette covered with vesicles. Higher temperatures frequently result in a slimy type of growth, a phenomenon common with this type of organism. Type A has been found on all satisfactorily fermenting cigar-leaf tobacco examined and is present in great numbers during the active stage of the fermentation. Without doubt this type is of significance in the fermentation process. It is probably a variant of *B. subtilis*.

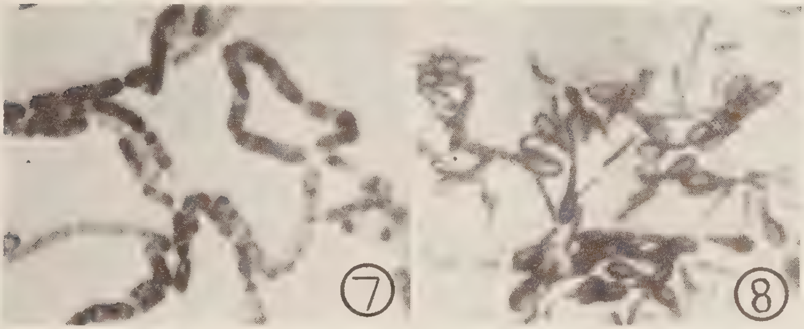

Fig. 7.—Microphotograph of a typical member of the *Bacillus megatherium–cereus* group. (950 X)
Fig. 8.—Microphotograph of a typical member of the genus *Clostridium* isolated from an early stage of bulk rot. (950 X)

Type B has also been found on most fermenting tobacco examined but usually in smaller numbers than Type A. This form differs from the other three types in ability to ferment raffinose and growth on potato, which is rapid, smooth and pink in color. Activity in litmus milk is similar to that of A and C; liquefaction of gelatin and growth on ferric ammonium citrate similar to that of C and D. Although this type bears a certain relation to *B. subtilis* in many respects it more closely resembles *B. vulgatus* and might be considered a variant of the latter. Gelatin liquefaction of these organisms is shown in figure 9.

Type C bears a certain resemblance to *B. subtilis* but in some respects more closely approximates *B. mesentericus*. This form grows well at 45° C while growth at 55° C is variable. It is characterized in particular by growth on potato which is similar to that of *B. mesentericus*. It has been found on all cigar-leaf tobacco examined and is usually predominant during the period of active fermentation. It is seldom found in the late stages of fermentation. Type D is the predominant type during the final stages of the fermentation of cigar-leaf tobacco from Ohio and Wisconsin. More

than half of all isolations made at that phase have been of this type when the tobacco was produced in Ohio or Wisconsin. This type has not been isolated from Pennsylvania crops. It differs from the other three types in its activity in milk, completely digesting the milk without coagulation within a period of 48 hours. The character of the growth on potato also differs from the other types. The temperature range of this type is not as wide as in the case of the other three. It grows best at 37° C, very little at 45° C and fails to grow at 55° C. It is probably a variant of *B. vulgatus*.

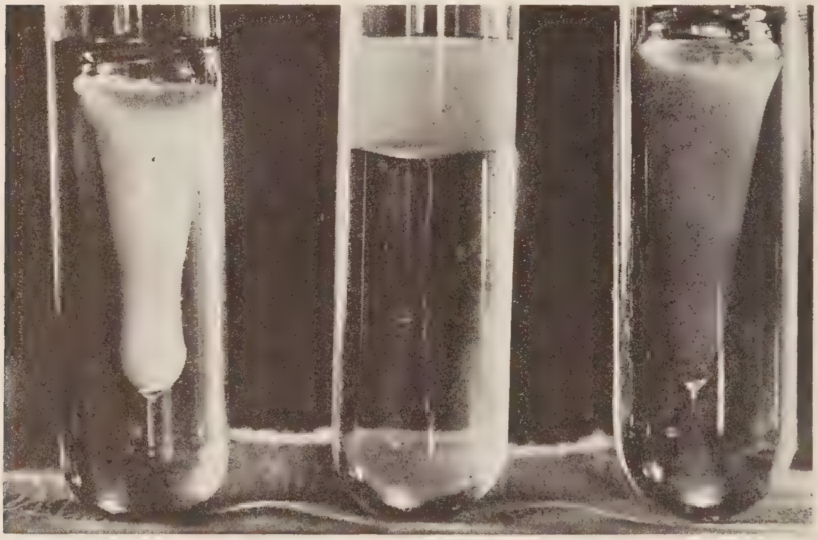

Fig. 9.—Gelatin liquefaction by aerobic spore-formers isolated from tobacco. From left to right, liquefaction typical of the *B. megatherium* group, Type A of the *B. subtilis–mesentericus–vulgatus* group, Type B of the same group. Types C and D react as does B.

That these four types are distinct is open to question. The characteristics exhibited at the time of isolation have been retained in the laboratory for more than a year. Type A differs sufficiently from the others to be set apart. The other three might be combined in one or two types.

From the data collected in this laboratory the conclusion must be drawn that the significance of the *B. subtilis–mesentericus–vulgatus* group in the fermentation of cigar-leaf tobacco is great. Reproduction to the extent noted in this group cannot be accomplished without important modification of the substrate.

The *B. brevis* group has been encountered less often than either of the other two groups of aerobic spore-formers. It has not been

found on fermenting Pennsylvania tobacco although isolated from tobacco grown in Ohio and Wisconsin. It is apparently never present in great numbers when compared with the numbers of the *B. subtilis–mesentericus–vulgatus* group. Reproduction must take place to some extent during the fermentation, however, as it has been isolated a number of times from the high dilutions plated during periods of active fermentation. This group differs from the *B. subtilis–mesentericus–vulgatus* group in inability to produce acetyl-methyl carbinol, very slow proteolysis of milk and inability to hydrolyze starch.

Schloesing *(14)* and Suchsland *(17)* described organisms on fermenting tobacco which were probably aerobic spore-formers. Davalos *(3)* found bacilli and described five types, some of which were undoubtedly identified with the *Bacillus subtilis–mesentericus–vulgatus* group. Behrens *(1)* specifically mentioned *B. subtilis* and Vernhout *(18)* definitely identified the tobacco organisms studied as members of the *B. subtilis* group. Koning *(8)* reported both gram-positive and gram-negative organisms and among the former described some of his types as related to *B. subtilis* and *B. vulgatus*. Schmidt *(15)* reported *B. mesentericus* on Ohio tobacco of good quality. Johnson *(6)* observed "large bacteria" and showed colony pictures which leave little doubt that members of the genus *Bacillus* were encountered by him. Giovannozzi *(4)* stated that *B. mesentericus, B. subtilis, B. megatherium* and *B. mycoides* were found by Rossi on fermenting tobacco.

It is concluded from the data presented in this and the preceding section that members of the genera *Bacillus* and *Micrococcus* are the microörganisms definitely associated with the fermentation of cigar-leaf tobacco. Figure 6 shows the numbers of these two genera present in a typical fermentation.

OTHER MICROÖRGANISMS

The presence of large numbers of aerobic spore-forming bacteria and cocci renders the use of a bacteriostatic agent necessary in any attempt to determine the presence of gram-negative bacteria upon tobacco of the cigar-leaf type. For this purpose crystal violet was chosen and various concentrations utilized in an effort to inhibit the gram-positive forms and permit the growth of the gram-negative bacteria.

From the cured leaf, particularly if the leaf is diseased, it is not difficult to isolate gram-negative rods of the type *Achromobacter* and *Flavobacterium*. Doubtless many of these gram-negative forms are members of the genus *Phytomonas* but the attempt has not been made to identify these isolations as such.

If fermentation progresses in a satisfactory manner it becomes very difficult to demonstrate the presence of gram-negative forms. The few colonies developing upon crystal violet agar are, as a rule, members of *Micrococcus* and *Bacillus* groups which the dye has failed to inhibit. Gram-negative bacteria cannot be assigned any significant rôle in the fermentation of cigar-leaf tobacco, on the basis of data accumulated in this laboratory. A few investigators, such as Koning *(8)*, have reported gram-negative organisms, but it is not clear that the type of tobacco examined was comparable to the cigar-leaf of Pennsylvania, Ohio and Wisconsin. That gram-negative forms multiply upon certain of the cigarette types is easily demonstrated.

The presence of members of the genus *Clostridium* can be shown by anaerobic incubation using crystal violet in the substrate to inhibit the facultative cocci and bacilli. These forms are either undergoing some multiplication in the normal fermentation or the spores are becoming more subject to germination on laboratory media as the fermentation progresses, as it becomes less difficult to demonstrate this type in the later stages of the fermentation. In incipient spoilage due to lack of aeration, and in actual rotting of the tobacco, these forms are found in large numbers. A microphotograph of a member of this genus isolated from tobacco is shown in figure 8. Oval, terminal spores seem characteristic of *Clostridium* found on tobacco.

Examination of tobacco in the early stages of rotting in the bulk fermentation reveals the presence of *Clostridium* of the cellulose-fermenting type. Isolation of these is accomplished in the medium of Omeliansky, pure cultures of types multiplying at 55° C having been obtained in this manner, following several enrichment transfers. Growth at 37° C of a similar type has been shown, but this has been found less frequently than has the thermophilic form.

Fungi on cured cigar-leaf tobacco.—The presence of fungi in large numbers is characteristic of cured cigar-leaf tobacco. More than 70 per cent of the fungi forming colonies on the laboratory media have been identified as members of the genera *Penicillium* and *Aspergillus,* the former genus being found more often than the latter. The several other colonies examined proved to be representative of a number of genera of the type commonly encountered in the examination of soil and plant material. Inasmuch as all these forms disappeared within a short time in the normal fermentation, it is thought that they have no important rôle in the process. Torula have occasionally been encountered on acid agar plates inoculated from fermenting tobacco, but never in significant numbers.

SUMMARY

Microörganisms of cured and fermenting cigar-leaf tobacco that were found in significant numbers have been isolated and studied in pure culture.

The predominant forms upon the cured leaf were bacteria of the *Bacillus megatherium* group and fungi of the genera *Penicillium* and *Aspergillus*.

Satisfactory fermentation is associated with a rapid rise in numbers of bacteria of the *Micrococcus candicans* type and the *Bacillus subtilis–mesentericus–vulgatus* group.

The predominant types upon the cured leaf apparently play little if any part in a satisfactory fermentation, viable fungi disappearing entirely in the early stages of the fermentation and bacteria of the *Bacillus megatherium* group failing to show any significant increase in number during the process.

The morphological characteristics of the cocci multiplying upon cigar-leaf tobacco, range from grapelike clusters of small spherical cells in early stages to large, oval-celled forms in later stages.

Many of these cocci resemble *Micrococcus candicans* in cultural characteristics. This type, however, is preceded by a somewhat more active type (physiological activity) and followed by a less active form.

The cultural characteristics of the bacteria of the *B. subtilis–mesentericus–vulgatus* group reproducing on fermenting tobacco allow division into four types. Three of these types are found in great numbers during the more active stages of the fermentation. The fourth is usually found in the final stages and may represent physiological changes induced in one or more of the three types present in great numbers in the earlier stages. One type might be characterized as a variant of *Bacillus subtilis,* the others might be considered variants of *Bacillus mesentericus* or *B. vulgatus,* possessing as they do some of the distinguishing characteristics of each of these species.

Members of the genus *Clostridium* are present on cured tobacco and may, under certain conditions, multiply rapidly, seriously affecting the quality of the finished product. Insufficient oxygen is responsible for the multiplication of these forms. The species of this genus found on fermenting tobacco include cellulose-decomposing types capable of reproduction at a temperature of 55° C. Rotting encountered during the fermentation may usually be attributed to members of this genus.

Gram-negative bacteria and non-spore forming rods have not been found reproducing in a satisfactory fermentation of cigar-leaf tobacco.

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